EXHIBIT B
Neurokinin-2 Receptor / ^{125}I-Neurokinin-A Binding Assay

MATERIALS USED IN THE ASSAY:
Membranes: Cloned neurokinin receptor subtype2, human, produced in CHO cells (Packard cat# 6110510)
Ligand: ^{125}I-Neurokinin-A (NKA) (NEN Life Sciences cat# NEX252)
Cold Neurokinin-A (NKA): BACHEM cat# 401192
Assay Buffer: 20mM HEPES (pH 7.4), 1 mM $MnCl_2$, 0.1% BSA
0.3% Polyethylenimine
Tissue grinder, glass, with teflon pestle
Stedfast Tissue Homogenizer
96 well round bottom polypropylene plates
filter plates (GF/C)
various pipeters, both single and multichannel
Packard Filtermate Harvester
Drying oven
Plate sealers, for top and bottom of filter plates
Scintillation fluid
Packard Topcount-NXT Microplate Scintillation Counter

METHODS:
Preparation of Assay Components:
The assay buffer was prepared by making a solution that was 20mM HEPES (pH 7.4), 1 mM $MnCl_2$, and 0.1% BSA.

The membranes, cloned neurokinin receptor subtype2, human, produced in CHO cells (Packard cat# 6110510), were thawed and, along with cold (stored at –80°C in 6 µl aliquots) NKA (BACHEM cat# 401192), kept on ice until the procedure was run.

The ligand ^{125}I-NKA (NEN Life Sciences cat# NEX252) was solubilized in sterile deionized water that has been degassed and purged with nitrogen and stored at –20°C. Unlabelled NKA was prepared in the same way.

Assay Procedure:
The volume of membrane stock solution to make 10 ml of 15 µg/ml for each assay plate was determined. The membranes were homogenized in approximately ½ of the final volume assay buffer, and then they were brought to the final volume with assay buffer.

The membranes (100 µl) were dispensed into all wells of a polypropylene round bottom 96 well plate.

The compounds to be screened were diluted with assay buffer in a 96 well deep well plate to obtain a final concentration of 60 µM. This entailed the addition of 4 µl of a 10 mM stock solution of test compound to 663 µl of assay buffer for a final concentration of 60 µM.

The radioligand was prepared by mixing 3.2 ml of 300 pM ^{125}I-NKA in assay buffer.

A volume of 60 µl of the 60 µM test compound solutions were transferred into columns 1-5 of a premix plate which was a round bottom 96 well polypropylene plate. Column 6 of the premix plate was reserved for controls, wherein 2 wells got 60 µl of assay buffer, 2 wells got 60µl of 300 nM unlabelled NKA for nonspecific binding, 2 wells got 60 µl of 60 nM known NK-2 binding control compound, a commercially available reference compound, and 2 wells were free, wherein other controls or 2 additional test compounds could be run. A volume of 60 µl of the 300 pM ^{125}I-NKA solution was transferred to the wells containing the test compounds and controls.

A volume of 50 µl of the solutions in the premix plate were transferred to the assay plate which contained the membranes, in duplicate. The plate was incubated for two hours at room temperature.

Harvesting Procedure:
A filter plate was pre-soaked in 0.3% polyethylenimine 30 min prior to filtering.

The assay plate was harvested into the filter plate, washed 5X with saline, and dried by placing in a drying oven for a minimum of 30 min. The bottom of the filter plate was sealed, and a volume of 25 µl of scintillation fluid was added per well. The top of the plate was then also sealed.

The plate was counted using a microplate scintillation counter, wherein the emissions in the 2.9 to 100KeV were counted.

Data Analysis:
The data for the 4 wells containing no inhibitors were averaged. The data for the 4 wells containing 50 nM unlabelled NKA were also averaged to provide a measure of nonspecific binding. The maximum specific binding was calculated by subtraction of the nonspecific binding from the average of the wells containing no inhibitors. The percent of control and percent inhibition were calculated using the formulas below:

Percent of Untreated Control = ((Test average-Nonspecific Binding)/Max) x 100

Percent Inhibition = 100 – Percent of Untreated Control